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(54) Monoclonal Antibody to HLI and its use for purifying HLI

(57) The present invention provides a monoclonal antibody which is directed against natural or recombinant human leukocyte interferon (HLI), characterised in that the antibody is of I_gG isotype, it has a γ_1 heavy chain, and it binds to recombinant HLI A and D but not to recombinant HLI B and F, which is suitable for use in purifying HLI, particularly by affinity chromatography.

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SPECIFICATION

Monoclonal antibody to HLI and its use for purifying HLI

The present invention is concerned with antibodies proteins, namely against human leucocyte interferon (HLI) which is natural or obtained by recombinant DNA technology.

HLI is possibly a valuable therapeutic for the treatment of neoplastic and viral illnesses. The evaluation of this substance and its possible development to a widely-used medicament is restricted by the difficulty of purifying and of characterising the active substance in the requisite manner. A test which could be carried out in less than 24 hours would be a valuable aid for the desired purification and the requisite characterization of HLI.

In the scope of the present invention it has now been found that for the purification of HLI in an excellent manner there is suitable a particular monoclonal antibody which is directed against leucocyte interferon which is natural or obtained by recombinant DNA technology.

According to the present invention, there is provided a monoclonal antibody which is directed against natural or recombinant human leukocyte interferon (HLI) characterised in that the antibody is of I_gG isotype, it has a γ_1 heavy chain, and it binds to recombinant HLI A and D but not to recombinant HLI B and F.

This antibody is suitable for use in purification of HLI, preferably by affinity chromatography. In a second aspect, the present invention provides a process for the purification of HLI characterised in that HLI-containing raw material is purified by affinity chromatography using the monoclonal antibody defined above. For the purification in a commercial scale the affinity should be high enough so that the interferon is practically quantitatively retained also with great volumes which pass the column with a not too low speed. With a single antibody this is not possible for all sub-classes of interferon. For this purpose a collection of monoclonal antibodies which individual members show a high affinity for particular sub-classes of interferon is especially suitable.

The preparation of the monoclonal antibodies is effected according to cell fusion technology known per se, in which the myeloma cells are fused with spleen cells of mice, which have been immunized with HLI. The hybridomas obtained secrete monoclonal antibodies against leucocyte interferon which is natural or obtained by recombinant DNA technology.

The purification of HLI by means of a monoclonal antibody can be effected according to methods known per se, whereby the affinity chromatography has been found to be particularly suitable. In this affinity chromatography Affi-Gel 10 (BioRad Laboratories, Richmond, California) Affi-Gel is a Registered Trade Mark. is preferably used as the carrier.

On the other hand, it has been shown that human leucocyte interferon obtained by recombinant DNA technology can be purified with a monoclonal antibody in accordance with the present invention in a simple manner such that it is usable for clinical trials.

In the following Examples, the present invention is illustrated with reference to the accompanying drawings, in which

Figure 1 is a graph showing the binding of various monoclonal antibodies to various types of HLI; and

Figure 2 is a graph showing the binding of various monoclonal antibodies to various sub-types of HLI.

In the Examples ® indicates a registered Trade Mark.

EXAMPLE 1

Manufacture of the monoclonal antibodies

A) *Interferon preparation*

Partially purified human leucocyte interferon (HLI) was available in sufficient amount for the immunization of the mice. 5 HLI preparations are used.

a) HLI γ(a) main peak of the fraction in accordance with step 8 of Table 3 of DOS No. 29 47 134 with an approximate purity of 10-15% (estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and specific activity in the antiviral test;

b) HLI α; c) HLI β; d) HLI γ; and e) HLI δ.

The HLI α, β and γ fractions consist in each case of mixtures of the individual (α_1, α_2), β_1, β_2, β_3) and (γ_1, γ_2, γ_3, γ_4, γ_5) species. These are shoulder fractions of the corresponding purified species, which are pooled from different preparations (see step 9 of Table 3 of the previously mentioned DOS). The HLI δ fraction is the shoulder of the δ-species. Besides the species α, β and γ on the Lichrosorb-diol® column (see step 8 of Table 3 of the previously mentioned DOS) there is occasionally seen a δ-fraction. This is purified as in step 9 in accordance with Table 3 of the previously mentioned DOS, whereby it is evident that the species δ shows no sub-species and is uniform. The purity of b), c) and d) is between 20 and 40% in accordance with specific biological activity and in accordance with sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The purity of e) is at most 5%.

Determination of interferon activity

The interferon activity is determined by means of the "Cytopathic-effect-inhibition" (CPE) test in accordance with U.S. Patent Specification No. 4,241 174 (Serial No. 963,256).

B) *Immunization of the mice*

3 eight weeks old Balb c/J female mice are firstly immunized with HLI γ(a) in Freund's complete adjuvant. Each mouse receives approximately 150 μg total protein containing 20-25 μg of interferon in 0.25 ml at 5 different positions (0.05 ml in each position): subcutaneously in the left and right iliac region and in the left and right axilliary region as well as an intraperitoneal injection.

53 days later a second immunization is carried out as follows: interferon HLI γ(a) is separated by preparative sodium dodecyl sulphate-polyacrylamide (15%) gel electrophoresis in three 0.6 x 11 cm cylindrical gels. Approximately 300 μg total protein in 0.3 ml (dialyzed against assay buffer) containing approximately 40-50 μg of interferon are loaded per gel. [This purification is effected essentially according to the method of Laemmli, U.K. (1970) Nature 227, 680-685]. After the electrophoresis, the gels are sliced into 2 mm thick discs. These are immersed for 10 minutes in 0.5 ml of phosphate-buffered sodium chloride solution (0.01 M potassium phosphate buffer, pH 7.3; 0.14 M sodium chloride) containing 0.1% Triton X*-100 in propylene test tubes. The buffer is investigated for interferon activity in serial dilution according to the CPE inhibition test. The discs 6 and 7 (numbered from the lower end of each gel) show the highest interferon activity (in each case approximately 25% of the activity of the total gel). In each case one disc with highest activity is finely sliced with a razor blade on a glass plate, transferred into a 1 ml syringe containing 0.2 ml of 0.15 M sodium chloride and injected into each mouse. The gel suspension is injected intraperitoneally, whereupon 0.2 ml of BCG (Bacillus Calmette-Guérin; Serum Institute Berne) are injected.

After 12 days, the serum of each mouse is investigated for interferon neutralization activity. The serum of mouse No. 3 shows a 50z neutralization 10 units/ml of HLI γ(a) at a dilution of 1:72 000, but does not neutralize the same concentration of crude HLI (< 0.1% purity) even at a dilution of 1:100. The mice No. 1 and No. 2 show neutralization titres of less than 1:1000 against HLI γ(a).

70 days after the second immunization mouse No. 3 receives on three successive days an intraperitoneal booster injection with a mixture of HLI α, β and γ containing approximately 50 μg of interferon and 15 μl of normal mouse serum as the carrier protein (in 0.1 ml of 0.15 M sodium chloride). 48 hours after the last injection the mouse is killed and the spleen is removed for the preparation of the monoclonal antibodies.

C) *Cell cultures and cell fusions*

The following materials and media are used. Iscove's modification of Dulbecco's modified Eagle medium (IMDMEM) is obtainable from Gibco. It is made up freshly with sodium pyruvate (1 mM), glutamine (1.5 mM), 2-mercaptoethanol (5×10^{-5}M), penicillin (100 units/ml) and streptomycin (100 μg ml). Complete HAT medium consists of thus-completed IMDMEM as well as hypoxanthine (10^{-4}M), aminopterin (4×10^{-7}M), thymidine (1.5×10^{-5}M) and 16% foetal calf serum. A 50% (w/v) solution of polyethyleneglycol 4000 (PEG 4000, Merck) in IMDMEM is prepared.

The fusion with a non-producing azaguanine-resistant myeloma cell line is carried out, with small modifications, according to the method of Stähli et al. in J. Immunol. Methods *32*, 297-304. In this fusion 48×10^6 nucleated cells are fused with 25×10^6 myeloma cells of the line FO (Fazekas de St. Groth and Scheidegger in J. Immunol. Methods *35*, 1-25 (1980). The spleen cells and myeloma cells are washed in serum-free IMDMEM. They are then re-suspended in the same medium, mixed in the above-mentioned ratio for the fusion and sedimented in 40 ml of serum-free IMDMEM in a 50 ml conical polypropylene test tube at 200 x g for 15 minutes, followed by complete sucking off the supernatant. To the cell sediment there are added dropwise with constant stirring 0.5 ml of 50% PEG 4000 in order to re-suspend and to disperse the cells. After approximately 90 seconds, 10 ml of serum-free IMDMEM are added dropwise at room temperature with constant stirring during 4-5 minutes. After a further 15 minutes without stirring, large cell clumps are separated by cautious pipetting with a 10 ml pipette. The fusion mixture is diluted to 250 ml with complete HAT medium and then placed in 240 "Costar cluster wells (1 ml/well)", which already contain 1 ml of complete HAT medium and 10^5 peritoneal mouse cells as the nutritive layer. The cultures are incubated in a 5% CO_2/95% air atmosphere at 85% humidity. The cultures are fed twice a week by replacing half of the medium (1 ml) with fresh HAT medium.

D) *Detection of interferon-specific hybridomas by a solid phase-antibody binding test (SABA)*

This method is adapted from the principle described by Catt and Tregear [Science *158*, 1570-1572 (1967)] and is suitable for the detection of hybridoma antibodies (see also Stähli et al in J. Immunol. Methods *32*, 297-304 (1980) as well as Kennet, R.H. in Current Topics in Microbiology and Immunology, Vol. 81, p. 77-91 (1978). Interferon preparations of HLI α, β, γ and δ are individually diluted in polypropylene test tubes with phosphate-buffered sodium chloride solution (PBS) to an end concentration of approximately 0.2 to 0.5 μg interferon/ml (approximately 1-3 μg total protein per ml; except for HLI δ where approximately 10-15 μg of total protein are present per ml), in order to coat therewith polyvinyl chloride microtitre plates (Cooke Laboratory Products Division, Dynatech Laboratories, Inc.) as in the case of Stähli et al. loc. cit.. 50 μl of the interferon solution is placed in each well and left for at least 4 hours at room temperature in a humid chamber or held at (sic) 4°C for several weeks. Before use the wells are filled with 3% bovine serum albumin (BSA) in PBS and left for 30 to 60 minutes in order to block all protein binding positions. The plates are then washed four times with PBS. 50 μl of supernatant of the hybridoma cultures are incubated in in each case two wells for at least 4 hours at room temperature. Control experiments (unspecific binding) are carried out on plates which are coated only with 3% BSA. After four-fold washing with PBS, there is added to each well

50 µl of sheep-anti-mouse Ig-antibody (purified by affinity chromatography and labelled with ^{125}I; 50,000 to 100 000 cpm per 20 to 30 ng of antibody in PBS containing 1% BSA) and it is incubated at room temperature for at least 4 hours. The plates are washed four times to five times with PBS and then divided into the individual wells, which are then tested for radioactivity in a gamma-scintillation spectrometer

E) *Results*

a) *Initial screening by antibody binding (SABA) against partially purified-HLI fractions*

152 of the 240 cultures show growth of hybridomas. The supernatants are tested for interferon-specific antibodies in the SABA test, when the cultures are approximately 20-30% confluent (12-29 days after the fusion). Antibody binding is tested parallel with HLI α, HLI β, HLI γ and HLU δ. Of the 152 cultures, 13 supernatants show high antibody binding against all four partially purified interferon preparations. The medium of four further hybridomas shows lower binding activity (3-4 times greater than unspecific binding) against all four interferon preparations. These cultures are discarded. The media of all other hybridomas (135 growing cultures) yield unspecific bindings against all four HLI preparations. Of the 13 specific hybridomas, 9 are characterized representative for the present invention. Their designation is LI-1, LI-2, LI-3, LI-5, LI-6, LI-7, LI-8, LI-9 and LI-12.

As is evident from Figure 1, the antibody binding of the 9 specific hybridomas shows a different behaviour to the 4 partially purified interferon preparations. LI-1, LI-2, LI-3, LI-5, LI-6, LI-7 and LI-8 show an identical characterization with the following binding sequence: HLI $\alpha <$ HLI $\delta <$ HLI$_\gamma <$ HLI β. For LI-9 the binding order is HLI $\delta <$ HLI $\gamma <$ HLIβ. LI-12 is different from all others.

b) *Antibody binding (SABA) against highly purified leukocyte interferon*

Six purified (≥ 80% pure) interferon species (see step 9 of Table 3 of DOS 29 47 134) are immobilized by adsorption on polyvinyl chloride microtitre plates. In this case there are concerned HLI α_1, α_2, β_2, γ_2 and γ_3 as well as HLI-K of the cell line KG-1 [Koeffler, H.P. et al. Science *200*, 1153-1154 (19780)], which is purifed in analogy to the steps 1 to 9 of Table 3 of the mentioned DOS. Since no clear separation into individual species was to be observed in the steps 8 and 9, the main fraction with the interferon activity was pooled. It accordingly represents a mixture of different species. The adsorption of interferon (50 µl/per depression) is carried out overnight with approximately 0.5 µg of interferon and 2 µg of BSA per ml of PBS followed by blocking off of the protein binding positions with 3% PBS as described previously.

The antibody binding with the culture medium of the 9 specific hybridomas against the 6 different, purified interferons is shown in Figure 2. There can again be differentiated various groups of antibodies with different characteristic binding behaviour. The group 1 embraces the antibodies LI-1 and LI-2, which have identical binding characteristics, but which do not recognize HLIγ_3 The group 2 embraces the antibodies LI-3, LI-5, LI-6, LI-7 and LI-8 with a possible differentiation of two sub-groups. Sub-group 2a (LI-3, LI-5 and LI-6) shows overall higher binding (Figure 1 and Figure 2) than sub-group 2b (LI-7 and LI-8), whereas the latter sub-group binds relatively stronger to purified HLI α_1 and HLI K than the first. According to the interferon binding behaviour of the remaining antibodies in accordance with Figure 2 LI-9 has the most characteristics of sub-group 2b. LI-12 appears, in turn, to be unique.

Having regard to the fact of the high antibody binding to different purified interferons it is very unlikely that any of these antibodies was not directed against interferon, but against contaminants. This is indicated for most of these antibodies also by the capability of neutralizing the biological activity of interferon. LI-1, LI-2, LI-3, LI-5, LI-6, LI-7, LI-8 and LI-9 annul the inhibition of the viral CPE on MDBK cells induced by interferon. The interferon neutralization is tested with partially purified HLI γ(a) and/or purified HLI K, HLIγ_1, HLI α_2 and crude leucocyte interferon. None of the antibodies was in a position to neutralize crude leucocyte interferon. LI-12 neutralized none of the tested interferons (see Table 1 for this).

2) *Isotypes of the heavy and light chains of the monoclonal interferon antibodies*

Monoclonal antibodies of hybridoma culture medium are firstly bound to interferon-coated microtitre plates as has been described previously for the SABA test. After washing, they are incubated with isotype-specific rabbit anti-mouse Ig antiserum (Nordic). For the detection there is used goat anti-rabbit IgG, which is labelled with horseradish-peroxidase. As the enzyme substrate there are used 2,2'-azino-di-(3-ethyl-benzothiazoline sulphate) and hydrogen peroxide. The results are compiled in Table 1, second column. Seven of the tested antibodies have the immunoglobulin chains γ_1/k, one has γ_{2b}/k and one has µ/k. All seven γ_1/k antibodies as well as also the γ_{2b}/k neutralize interferon, whereas the µ/k does not neutralize interferon, as is likewise evident from Table 1.

The ability of the antibodies according to the present invention to bind with high affinity to interferons obtained by recombinant DNA technology is evident from Table 3.

3) *Cloning and stabilization of the interferon-specific hybridomas*

For the preparation of stable hybridoma lines, all 9 specific hybridomas are cloned by limiting dilution in microtitre plates with mouse-peritoneal cells as the nutritive layer [Hengartner H. et al. in Current Topics in Microbiology and Immunology, Vol. 81, 92-99 (1978)]. The cloning was begun at the point in time of the transfer of the original cultures into flasks or shortly thereafter. The clones were tested against HLI β with he SABA test. The results of this first cloning of the 9 hybridomas is shown in Table 2.

From each hybridoma there are intensively cultivated two to four strongly positive clones and not only injected i.p. into mice for ascites production but also frozen. In most cases the selected clones of a hybridoma are pooled for simplification and reduction of work. From all 9 hybridomas there is obtained ascites liquor for the production of antibodies on a large scale. Ascites cells are likewise transferred into cultures and frozen.

4) *Grouping of the antibodies according to their interactions with HLI*

The main group of the seven hybridomas, which produce γ_1/k immunoglobulin, can be divided into two groups on the basis of their antibody interaction with purified interferons. The first group (LI-1 and LI-2) does not recognize purified HLI γ_3, whereas the second group (LI-3 and LI-5 to LI-8) recognizes HLI γ_3. The relative, quantitative binding of the monoclonal antibodies to six different purified interferons (see Figure 2) and the difference between neutralizing and non-neutralizing antibodies yields information for the differentiation of different epitopes (antigenic determinants) of HLI and for the demonstration of structural differences between different purified interferons. From the binding behaviour of the antibodies (see Figure 2) it is clear that LI-1 and LI-2 recognize a different structure than all other antibodies and that HLI γ_3 is structurally different from all other tested interferons, since it does not exhibit the epitope recognized by LI-1 and LI-2. LI-12, a non-neutralizing antibody, recognizes with approximately the same, although perhaps lower affinity, a less variable determinant which is present in all tested interferons. The results in respect of the antibody binding and of the interferon neutralization indicate that at least three different epitopes of HLI can be recognized and defined by the collection of the monoclonal antibodies.

EXAMPLE 2

Purification of leucocyte interferon obtained by recombinant DNA technology (Interferon A, c.f. Goeddel et al. Nature, vol. 20, March 1981).

a) *pre-purification of the human leucocyte interferon obtained by recombinant DNA technology*

All steps are carried out 4°C. Frozen bacteria cells (1 kg) are broken up by pressure disintigration. The extract is worked up according to the standard method, i.e. by polyethyleneimine (Polymin P) and ammonium sulphate precipitation, followed by a dialysis of the 65% ammonium sulphate precipitate against 25 mM Tris HCl, pH 7.8; 0.01% thiodiglycol; 0.1% Triton X-100*; 10 µM phenyl fluoride. A centrifugation follows for the removal of insoluble constituents.

b) *Preparations of the immunoadsorbent*

Purified antibody solution containing LI-8 is dialyzed at room temperature against 0.2 M sodium carbonate/0.3 M sodium chloride buffer. The dialyzed solution is centrifuged at 20 000 x g for 15 minutes in order to remove insoluble material. The protein concentration is adjusted to about 25 mg/ml with the above-mentioned buffer. Affi-gel 10 (BioRad Laboratories, Richmond, California) is washed three times with ice-cold isopropanol on a sintered glass filter and then washed three times with ice-cold distilled water. The gel is transferred into plastic test tubes and sedimented by brief centrifugation. The supernatant is sucked off. The gel is mixed with an equal volume of purified antibody solution and rotated head over foot at 4°C for 5 hours. After the reaction, the gel is centrifuged and then washed twice with buffer (0.1 M $NaHCO_3$/0.15 M NaCl) in order to remove unbound antibody. Protein determination of the combined wash-water shows that about 24 mg of antibody were bound per ml of gel.

c) *Affinity-chromatographical purification of the pre-purified leucocyte interferon obtained by recombinant DNA technology*

The solution obtained in step a) (700 ml containing 37 g of protein) is placed at 50 ml/h on the immuno-absorbent column (2.5 x 3.5 cm; 17 ml bed volume containing about 400 mg of purified monoclonal antibody LI-8) equilibrated with PBS. The column is washed with 20 column volumes of buffer (0.5 M NaCl; 0.02 M Tris. HCl, pH 7.5; 0.2% Triton X-100*), then the column is washed with 5 column volumes of 0.15 M NaCl, 0.1% Triton X-100* and then eluted with 0.2 M acetic acid, 0.15 M NaCl, 0.1% Triton X-100* (pH 2.5). The interferon activity is eluted in about 30 ml in an average concentration of approximately 1 mg protein/ml. This solution is adjusted to pH 4.5 with 1 M Tris base and diluted four-fold with water. The sample is loaded on to a carboxymethylcellulose (CM 52 Whatman*) column (1.3 x 3 cm), which is equilibrated with 0.1 M ammonium acetate (pH 5.0). The column is washed with 20 ml of 0.1 M ammonium acetate (pH 5.0) and the interferon is eluted with 20 ml of 0.5 M ammonium acetate (pH 5.0). The result of the purification is reproduced in Table 4.

TABLE 1

Epitope	Monoclonal antibody	Isotypes	Chains	Neutralization
I	LI-1	IgG	γ_1/k	Yes
	LI-2		γ_1/k	Yes
II	LI-3		γ_1/k	Yes
	LI-5		γ_1/k	Yes
	LI-6		γ_1/k	Yes
	LI-7		γ_1/k	Yes
	LI-8		γ_1/k	Yes
	LI-9		γ_{2b}/k	Yes
III	LI-12	IgM	μ/k	No

TABLE 2

Culture	Cells per 96 "wells"	Tested clones or "wells"	Number positive	Number negative
LI-1	100	6	6	0
LI-2	100	11	6	5
LI-3	100	7	7	0
LI-5	100	13	13	0
LI-6	100	12	12	0
LI-7	100	10	8	2
LI-8	100	9	8	1
LI-9	100	10	8	2
LI-12	100	4	0	4
LI-12	300	48	6	42

TABLE 3

	Antibody						
Interferon	LI-1	LI-3	LI-5	LI-6	LI-7	LI-8	LI-9
A	+	+	+	+	+	+	+
B	+	−	−	−	−	−	−
D	−	+	+	+	+	+	+
F	−	−	−	−	∓	−	−

Legend:
The interferons designated as A-F are those obtained by the DNA recombinant technology and described in the publication of David V. Goeddel et al. in Nature Vol. 290, March 1981. A + designates that a given interferon was completely absorbed, a ∓ partially absorbed and a − not absorbed by the columns loaded with the respective antibody.

TABLE 4

Step	Volume (ml)	Total Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Ammonium sulphate precipitation	700	37,100	7.4×10^9	2×10^5	1.0	100
Antibody column pool	30	30	7×10^9	2.3×10^8	1150	95
CM-52	20	20	6×10^9	3×10^8	1500	81

The specific activity was determined according to the CPE inhibition procedure mentioned earlier. In accordance with sodium dodecyl sulphate-polyacrylamide gel electrophoresis the interferon after the immunoadsorption step is over 90% pure. According to the CM-52 column it appears to be practically homogeneous.

CLAIMS

1. A monoclonal antibody which is directed against natural or recombinant human leukocyte interferon (HLI), characterised in that the antibody is of I_gG isotype, it has a γ_1 heavy chain, and it binds to recombinant HLI A and D but not to recombinant HLI B and F.
2. Use of the antibody of claim 1 for the purification of HLI.
3. A process for the purification of HLI characterised in that the HLI-containing raw material is purified by affinity chromatography using the monoclonal antibody of claim 1.
4. A process for the purification of HLI substantially as hereinbefore described with reference to Example 2.

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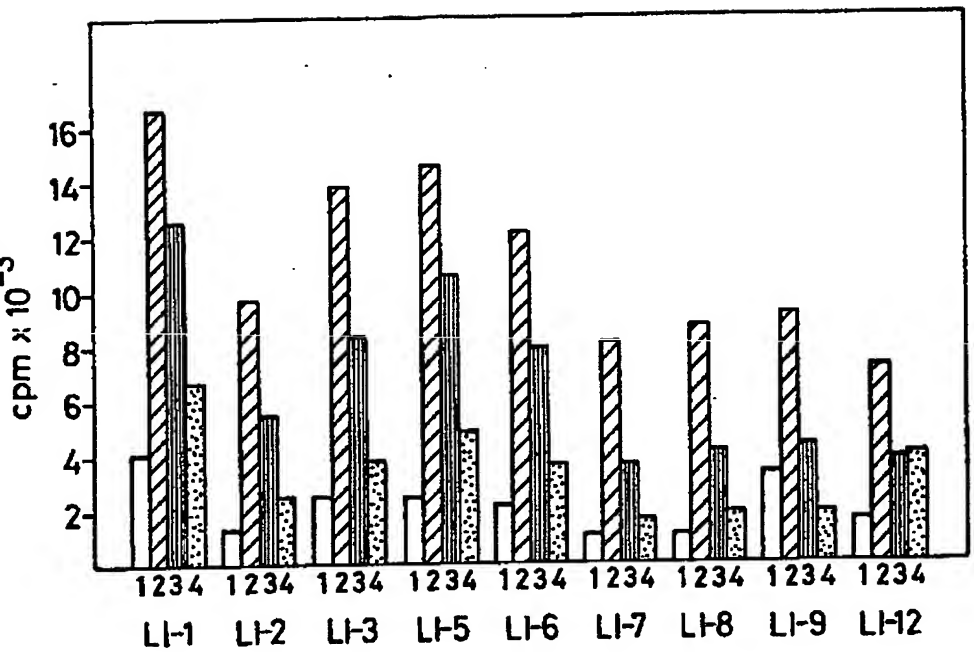


1 = IFL α

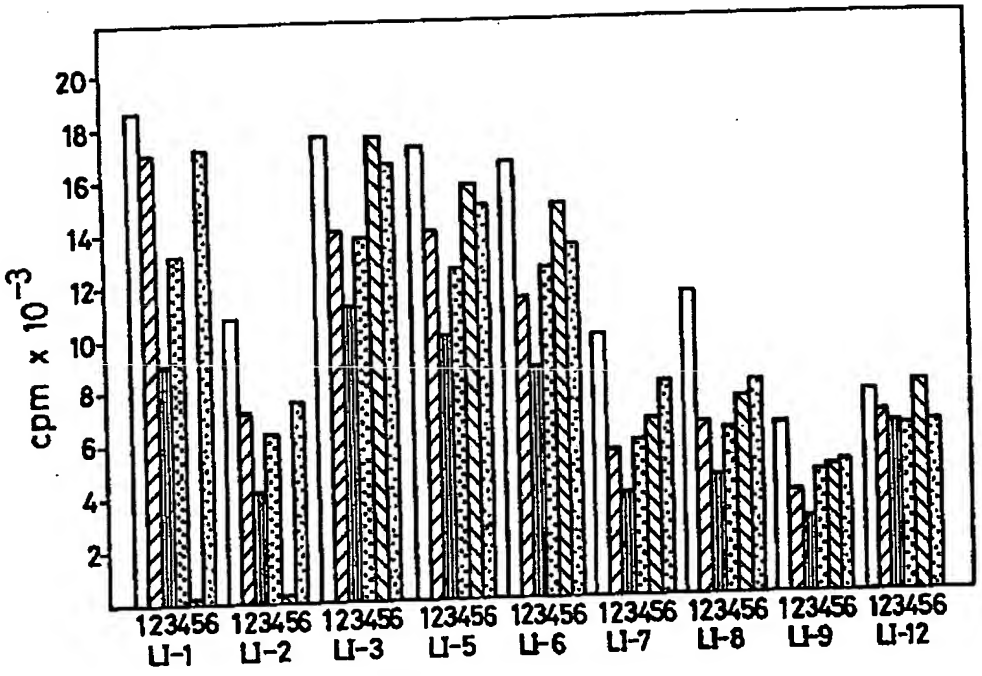
2 = IFL β

3 = IFL γ

4 = IFL δ

cpm = specific binding per test (after deduction of 300-400 cpm of non-specific binding against BSA; LI-12 non-specific binding about 1200 cpm)

FIG. 1



1 = IFL α_1

2 = IFL α_2

3 = IFL β_2

4 = IFL γ_2

5 = IFL γ_3

6 = IFL K

cpm = specific binding per test (after deduction of 300-400 cpm of non-specific binding against BSA; LI-12 non-specific binding about 1200 cpm)

FIG. 2